Author’s response to reviews

Title: Prospective Multicenter Randomized Patient Recruitment and Sample Collection to Enable Future Measurements of Sputum Biomarkers of Inflammation in an Observational Study of Cystic Fibrosis

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Point by point response

Editor Comments

- Please double check the author list on the manuscript and in the editorial system and ensure it is the same. Also please check that all authors a mentioned (with initials) in the author contribution section of the declarations.

We double checked the author list comparing the manuscript with the online system. There were two names in the manuscript byline missing from the submission website system. I will correct this at the time of resubmission by inserting Yanping Li and Jerimiah Lysinger into the author list in the submission system.
Every author is mentioned by initials at least once in the author contribution section of the declarations.

- Please move List of Abbreviations after the Conclusions section

We moved the list of abbreviations as asked.

- Please provide Conclusions section

We added a header to identify the Conclusions section.

- Under "Ethics approval and consent to participate", you didn't mention whether patients gave their consent to participate in the study. We also would require information on whether written or verbal consent was sought.

At the end of the Ethics approval and consent to participate section, we added, “All patients younger than 18 provided assent with informed written consent provided by their parents or other legal guardians. Adult patients gave informed written consent on their own behalf.” We additionally modified one sentence in the “Enrollment” section of the Methods to read, “We enrolled patients after written informed consent only when judged clinically stable by enrolling investigators.”

Comments from Dr. Emer Reeves

My main concern on this study is the limited information provided on sputum collection and processing. As sputum samples are intended for biomarker identification and inaccuracies for HMGB-1 measurements are reported, it is vital that further information on how samples were processed is included.

We extracted and summarized the steps for sputum processing from Additional File 5, the study protocol, Additional File 6, the written instructions and worksheet for processing sputum and Additional File 7, the video instructions for processing sputum and added the summarized instructions to the Methods, Sputum Collection, Processing and Shipping section. The new text reads,

“In accordance with Therapeutic Development Network standard operating procedure,40 we allowed one hour to collect sputum, transport from collection point on ice and initiate laboratory processing with an additional 1-3 hours to complete processing. We further specified that during the initial hour, collection time was limited to 20 minutes and 40 minutes were allowed to transport specimens on ice and initiate laboratory processing.”
Samples were collected in 50 ml conical tubes supplied by the central study coordinators at the University of Utah. Following collection, the sample was placed on ice and transported to the local processing laboratory. Sputum samples were weighed, diluted 1:1 with Hanks Buffered Saline Solution (Sigma, St. Louis, MO) and vortex mixed for 1 minute. A sterile disposable pipette was used to transfer 0.25 ml of the vortex mixed sample to a 1.8 ml tube containing Streck Solution (Streck Inc. La Vista, NE); contents were mixed using the pipette, and the tube was sent via Fedex (Memphis, TN) to the U of Utah for cell counts and differentials. The remainder of the vortex mixed sputum sample was centrifuged for 20 minutes at 2,800 g at 4 °C to produce a top lipid layer, a middle aqueous layer and a bottom pellet layer. The layers were carefully separated by transfer pipettes; all personnel were trained to separate the layers in a way to avoid contamination of the aqueous fraction by the other two layers.

The lipid layer was carefully transferred to a new 1.8 ml cryovial, labeled with the participant study identification number (ID), collection date, fraction identifier (L) and sample number. The aqueous layer was divided in two. The first half was diluted with HBSS (Sigma) 1:1, vortex mixed for 10 seconds, aliquoted and labeled with participant study ID, date, fraction identifier (SA), sample number and aliquot number. The second half was diluted with protease inhibitor cocktail (Sigma) 1:1, vortex mixed for 10 seconds, aliquoted and labeled with participant study ID, date, fraction identifier (SB), sample number and aliquot number. The pellet was transferred to a 1.8 ml cryovial and labeled with participant study ID, date, fraction identifier (P), sample number and aliquot number.”

The authors should compare their techniques of sputum sampling and processing for different barkers with the most recent published advances in this field.

We added the following sentences as the new 3rd paragraph of the Discussion in a new paragraph just preceding the modified paragraphs on limitations. The new paragraph includes three new citations that provide context for our current effort,

“Our method of sputum sample processing repeats the method we used to discover associations between HMGB-1 and pulmonary exacerbations and survival.25 In that effort, we modified the standard operating procedures of the Therapeutic Development Network40 by avoiding the use of dithiothreitol (DTT) in order to optimize detection of some inflammatory biomarkers.53 Other studies of the effects of avoiding DTT and adding protease inhibitors found that biomarker detection was improved in both cases.53–56”

Comments from Dr. Ralf Schubert

However, the title of the manuscript is misleading because the authors do not show and interpret biomarkers and the method to ship and analyze sputum samples seems to be not ready to use.

We respond in two parts as follows.

... the title of the manuscript is misleading because the authors do not show and interpret biomarkers…
We changed the Title to, “Prospective Multicenter Randomized Patient Recruitment and Sample Collection to Enable Future Measurements of Sputum Biomarkers of Inflammation in an Observational Study of Cystic Fibrosis,” which we think makes clear that the measurements of the biomarkers themselves are in the future relative to the report. We retain the key elements describing the type of study and the objective of the overall study.

We altered the Running Head to match, emphasizing recruitment and sample collection: “Recruitment and Sample Collection for a Prospective Randomized Observational Study in CF.”

In addition, we modified the last sentence of the Abstract to better indicate that this is a study that has not yet completed the measurements, analysis and interpretation of biomarker results:

“These findings will plausibly make future interpretations of quantitative measurements of inflammatory biomarkers generalizable to sputum-producing patients in the CFFPR.”

...the method to ship and analyze sputum samples seems to be not ready to use.

The sputum samples have completed collection, and the processing of the samples was briefly reported in the original manuscript, but not in great detail. For the revision, we clarify and expand on the entire processing methodology used. We summarize the material in Additional Files 5, 6 and 7 to add the paragraphs quoted above in response to Dr. Reeves’ first comment. The effect is that readers need not refer to the Additional Files to get a detailed overview of the sputum processing methods.

In addition, the method displays fundamental flaws in its performance eg. samples should be processed within 1-2 hours after collection, and at least 400 cells should be counted for cell Differentiation.

We agree with Dr. Schubert that rapid processing is best in accordance with published literature.

In the revision, we clarify the procedures. Our process allowed up to 20 minutes for patients to physically produce sputum. We required that sputum processing be started within the first hour after the start of collection and the entire process to be completed within 4 hours. While we wanted immediate processing for all samples, this was not always feasible due to the physical placements of clinics and laboratories. These were typically in separate buildings which required transport either by walking or driving with the sample kept on ice.

However, while we required the total time to completion of processing to be 4 hours, we note that the average time was under 1 hour. We added that information to the Results in the second paragraph of the section, Sputum Samples.

We added a reference to what are now known as the Standard Operating Procedures for the Therapeutic Development Network (TDN) of the CF Foundation. These procedures specify times up to 4 hours for completion of sputum processing. These procedures have been used for multiple studies in CF over the last 20 years.
We clarify our procedure for cell count and differentiation. We originally reported the lowest number of cells counted as being about 80, which was confusing and suggested that number as the lower cutoff; however, that was an outlier due to low cellularity in a small pediatric specimen. Our intended overall procedure counted between 200 and 500 cells to derive a differential. We agree that the lower number is still below the general guidelines that can be found in the literature for evaluation of sputum and bronchoalveolar lavage samples, but it is in accordance with the guidelines published for TDN studies on how to process sputum obtained from patients with CF intended for study of inflammatory biomarkers. Because these guidelines were so specific to the intent of our study, we followed them instead of more general sputum processing guidelines. In addition to the text clarifications, for thoroughness, we repeated cell differentials during revision and slightly revised the reported overall differentials and added information about the total cell counts performed on the specimens. We added the reference to the section on cell differentiation in the results section Sputum Samples, and the final revised section in question reads,

“The average differential of 36% neutrophils, 30% lymphocytes, <1% eosinophils and 34% other cells was based on 108 out of 112 samples. A few samples had inadequate preservation in transport or sparse cellularity thus less than 200 cells were counted, but we counted 200-500 cells for the rest of the samples in accordance with Therapeutic Development Network standard operating procedures for samples from patients with CF collected for inflammatory marker measurements (mean 250 cells counted/sample, range 41-541).40”

New reference: